Involvement of the *Haemophilus ducreyi gmhA* Gene Product in Lipooligosaccharide Expression and Virulence

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The lipooligosaccharide (LOS) present in the outer membrane of *Haemophilus ducreyi* is likely a virulence factor for this sexually transmitted pathogen. An open reading frame in *H. ducreyi* 35000 was found to encode a predicted protein that had 87% identity with the protein product of the *gmhA* (isn) gene of *Haemophilus influenzae*. In *H. influenzae* type b, inactivation of the *gmhA* gene caused the synthesis of a significantly truncated LOS which possessed only lipid A and a single 2-keto-3-deoxyoctulosonic acid molecule (A. Preston, D. J. Maskell, A. Johnson, and E. R. Moxon, J. Bacteriol. 178:396–402, 1996). The *H. ducreyi* *gmhA* gene was able to complement a *gmhA*-deficient *Escherichia coli* strain, a result which confirmed the identity of this gene. When the *gmhA* gene of *H. ducreyi* was inactivated by insertion of a *cat* cartridge, the resultant *H. ducreyi* *gmhA* mutant, 35000.252, expressed a LOS that migrated much faster than wild-type LOS in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When the wild-type *H. ducreyi* strain and its isogenic *gmhA* mutant were used in the temperature-dependent rabbit model for dermal lesion production by *H. ducreyi*, the *gmhA* mutant was found to be substantially less virulent than the wild-type parent strain. The *H. ducreyi* *gmhA* gene was amplified by PCR from the *H. ducreyi* chromosome and cloned into the pLS88 vector. When the *H. ducreyi* *gmhA* gene was present in *trans* in *gmhA* mutant 35000.252, expression of the *gmhA* gene product restored the virulence of this mutant to wild-type levels. These results indicate that the *gmhA* gene product of *H. ducreyi* is essential for the expression of wild-type LOS by this pathogen.

*Haemophilus ducreyi*, a gram-negative coccobacillus, is the etiologic agent of the sexually transmitted disease known as chancroid. This ulcerogential disease is endemic in areas of Africa, Asia, and Latin America (63), and a resurgence of chancroid has been seen in the United States since early in the 1980s (63). With the finding that chancroid as well as other genital ulcer diseases are significant risk factors for the transmission of the human immunodeficiency virus (63), there has been renewed research effort to elucidate the pathogenic mechanisms and virulence factors of *H. ducreyi*.

To date, several putative virulence factors of this organism have been identified; these include a cell-associated hemolysin with cytotoxic activity (2, 43), a soluble cytotoxin (12, 50), a hemoglobin-binding outer membrane protein (18, 61), a novel pilus (6), and a copper-zinc superoxide dismutase (52). In addition, gene products which may directly or indirectly regulate expression of LOS have been described (9, 21, 60), and while at least four different genes whose encoded protein products are directly or indirectly involved in *H. ducreyi* LOS expression have been cloned and sequenced (21, 60, 66), only one of these has been tested by mutant analysis in relevant in vitro and in vivo systems. An *H. ducreyi* mutant defective in the expression of an RfaK homolog was shown to exhibit a reduced ability to attach to and invade human keratinocytes in vitro (21). Virulence testing of an independently constructed *H. ducreyi rfaK* (i.e., *lbgB*) mutant in the temperature-dependent rabbit model for experimental chancroid (49) yielded equivocal results (60).

In the present study, a gene (*gmhA*) encoding a phosphoheptose isomerase essential for the expression of wild-type LOS by *H. ducreyi* 35000 was identified and shown to complement an *Escherichia coli* *gmhA* mutant. Inactivation of the *H. ducreyi* *gmhA* gene resulted in the expression of a truncated LOS molecule. In addition, the isogenic *gmhA* mutant exhibited significantly reduced virulence in an animal model for experimental chancroid. Provision of the wild-type *H. ducreyi* *gmhA* gene in *trans* in the isogenic mutant restored both wild-type LOS expression and virulence.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Some of the bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) medium (51). LB medium was supplemented, when appropriate, with ampicillin, chloramphenicol, or kanamycin at a final concentration of 100, 50, or 30 μg/ml, respectively. *H. ducreyi* strains were grown on chocolate agar (CA) plates containing 1% (vol/vol) IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) as previously described (49). *H. ducreyi* strains were grown in a humidified atmosphere of 95% air–5% CO2 at 33°C. CA plates supplemented with chloramphenicol at a final concentration of 2 μg/ml were used for mutant selection. For complementation studies involving *H. ducreyi*, kanamycin was included in CA at a final concentration of 30 μg/ml. In growth studies, *H. ducreyi*...
strains were grown at 33°C with slow shaking in a water bath; growth was monitored by measurement of culture turbidity. A modified Haemophilus somnus liquid medium (29) was used in these growth studies and consisted of sterilized Columbia broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.1% (wt/vol) Tris base, 1% (vol/vol) IsoVitaleX, and heme at a final concentration of 25 μg/ml.

Recombinant DNA techniques. Standard techniques, such as restriction enzyme digests, ligation, transformation, electroporation of E. coli strains, and plasmid purification, have been described elsewhere (4, 51). PCR was done with Taq polymerase (Promega, Madison, Wis.), 100 ng of each primer (see below), 2.5 mM MgCl₂, and deoxynucleoside triphosphates at 200 μM each. Twenty-nine cycles at an annealing temperature of 55°C were performed for each PCR (62). Boiled bacterial cell preparations (27) or chromosomal DNA purified from H. ducreyi DNA insert in pHD251 were used as the template for PCR.

Chromosomal DNA preparation. Chromosomal DNA was purified from an overnight culture of H. ducreyi by use of a modification of a method previously described (5). The bacteria were scraped from a single CA plate into 3 ml of sterile phosphate-buffered saline (PBS) solution, and the mixture was centrifuged to pellet the bacteria. The bacterial pellet was resuspended in 2 ml of 10 mM Tris-HC1 containing 1 mM EDTA (pH 8.0), and 150 μl of 10% (wt/vol) sodium dodecyl sulfate (SDS) was added. The mixture was incubated at room temperature for 5 min and then at 37°C for 1 h. Next, 200 μl of RNase (10 mg/ml) was added, and the mixture was incubated at 37°C for 1 h, and then 20 μl of 3 M NaCl and 20 μl of protease K (10 mg/ml) were added. The mixture was incubated at 56°C for 2 h. One phenol extraction, two phenol-chloroform extractions, and one chloroform extraction were performed in succession as described previously (5). The linearized form of pHD251 was used to electroporate E. coli HB101. Plasmid pHD252 was purified by cesium chloride density gradient centrifugation and linearized by digestion with BsiI. The linearized form of pHD252 was used to electroporate H. ducreyi 35000 (24), and transformants were selected on CA plates supplemented with chloramphenicol.

E. coli HB101 was used for propagating pHD252 prior to the use of this DNA for electroporation of H. ducreyi. For reasons that remain to be determined, mutated H. ducreyi DNA inserts from plasmids propagated in some other E. coli strains, including DH5α, do not allow successful electroporation of H. ducreyi (data not shown).

Construction of an H. ducreyi genomic library in pWK30. Chromosomal DNA purified from H. ducreyi 35000 was partially digested with SacI. Fragments larger than 6 kb were purified by sucrose density gradient centrifugation, ligated into the BamHI site of pWK30 (67), and used to transform E. coli DH5α.

Nucleotide sequence analysis. Nucleotide sequence analysis was performed with a model 373 automated DNA sequencer (Applied Biosystems, Foster City, Calif.). Both strands of the 1.6-kb H. ducreyi DNA insert in pH251 were sequenced in their entirety, as were both strands of the same 1.6-kb region in pH252. DNA sequence information was analyzed through the National Center for Biotechnology Information by use of the BLAST network service to search GenBank (3) and with MacVector sequence analysis software (version 6; Oxford Molecular Group, Campbell, Calif.).

Construction of an isogenic H. ducreyi gmhA mutant. The oligonucleotide primers P1 (5′-GGGATCCCAAGAGTTCTTATCTTCACCAC-3′) and P2 (5′-CGGGATCCACGTTACCCATTCAGTTACCTTATG-3′) (see Fig. 2) were constructed with a BamHI site (underlined) based on information obtained from the nucleotide sequence of the H. ducreyi DNA insert in pH250. The use of P1 and P2 in PCR together with 400 ng of purified H. ducreyi 35000 chromosomal DNA yielded a 1.6-kb product containing the H. ducreyi gmhA open reading frame (ORF) and flanking DNA. This PCR product was digested with BamHI and then ligated into the BamHI site of pBluescript II KS+ + (pBS; Cloning vector; Amp′). pHD251 pBS with a 1.6-kb PCR-derived insert containing the H. ducreyi 35000 gmhA gene was digested with both PpuI and then ligated into the PvuI site within the gmhA gene (This study). pHD252 pCR2.1 Cloning vector capable of replication in H. ducreyi; Kan′ Sm′ Sul′ (16)

Plasmids pLS88 Cloning vector; Amp′
pCR253 pCR2.1 with a 1-kb PCR-derived insert containing the H. ducreyi gmhA gene (This study)
Spin columns (Boehringer). (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and purified on Quick Zealand White adult male rabbits were used in each experiment. These animals used in this study (49). These studies involving rabbits were approved by the Institutional Animal Care and Research Advisory Committee. Briefly, eight New Zealand White adult male rabbits were used in each experiment. These animals also used as a probe for Southern blot analysis. These two DNA probes were AACATCTGCTAATCCTGCC-3. The 1.4-kb oligonucleotide primers P5 (5'-TCTTGTGGCAATGGCGGTTC-3') (see Fig. 2). The 1.4-kb cat cartridge was ligated into the Pps10I site of pH252 to construct pH252. Plasmid pCR253 is pCR2.1 with a 1-kb PCR product containing the gmhA gene and flanking DNA; the small boxes on the ends of this insert represent nucleotides involved in cloning into the pCR2.1 vector. Plasmid pl8253 is pl808 with the aforementioned 1-kb PCR product cloned into the EcoRI and SacI sites of this vector.

Southern blot analysis. Purified H. ducreyi 35000 chromosomal DNA was digested to completion with AflIII or HindIII, subjected to electrophoresis in a 0.7% (wt/vol) agarose gel, transferred to nitrocellulose paper, and probed by Southern blot analysis as previously described (51). A DNA probe comprising 330 bp internal to the H. ducreyi gmhA gene was constructed by PCR with the oligonucleotide primers P5 (5'-TCCTGTGGCAATGGCGGTTC-3') and P6 (5'-AACATCTGCTAATCCTGCC-3') (see Fig. 2). The 1.4-kb cat cartridge was also used as a probe for Southern blot analysis. These two DNA probes were radiolabeled with [32P]dCTP by use of a random-primer DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and purified on Quick Spin columns (Boehringer).

Analysis of H. ducreyi LOS and E. coli LPS. Whole-cell lysate preparations of E. coli and H. ducreyi strains were treated with proteinase K as previously described (45), and the LOS or lipopolysaccharide (LPS) in these preparations was resolved by Tricine-SDS-polyacrylamide gel electrophoresis (PAGE) (35). The resultant gels were either stained with silver (64) or transferred to nitrocellulose paper, and identified by Western blot analysis. The in the scoring of the resultant lesions. Lesion characteristics were scored as described previously (49) with the following numeric values: 0, no change; 1, erythema; 2, induration; 3, nodule; and 4, pustule or necrosis. Lesion scores were recorded on days 2, 4, and 7 postinfection. On day 7 postinfection, material excised from lesions caused by injection of 10³ CFU of H. ducreyi was cultured on CA plates. Recovered organisms were subcultured onto CA supplemented with appropriate antimicrobial compounds to confirm the presence of therelevant antimicrobial resistance markers and were also tested by a colony blot radioimmunoassay with MAb 3E6 to confirm the LOS phenotype. Statistical analyses were performed as described previously (60, 61).

Nucleotide sequence accession number. The nucleotide sequence of the H. ducreyi gmhA gene was deposited at GenBank and assigned accession number AF045156.

RESULTS

Identification and nucleotide sequence analysis of the gmhA gene of H. ducreyi 35000. A recombinant clone (E. coli DH5α (pHD250)) derived from an H. ducreyi 35000 genomic library constructed in plasmid vector pWKS30 was observed to give rise to translucent and opaque colonies (data not shown). Nucleotide sequence analysis of 1.6 kb of DNA at one end of the 18-kb H. ducreyi DNA insert in pH250 detected an ORF (Fig. 1) which encoded a predicted protein with homology to the gmhA gene product of H. influenzae (8). Expression of this H. influenzae gene, also designated isn (47), is essential for both the synthesis of wild-type LOS by H. influenzae type b and
the expression of full virulence by this encapsulated pathogen (69). This 1.6-kb region of the DNA insert in pHD250 was found to contain two incomplete predicted ORFs and one complete predicted ORF (Fig. 1). The complete ORF, encoding the GmhA-like protein, was transcribed in the opposite direction from the other two ORFs (Fig. 1). Of the two incomplete ORFs, one (ORF A) encoded a protein with homology to a transcriptional regulatory protein (HI0410) of *H. influenzae* (20), and the other (ORF B) most closely resembled a hypothetical ORF (HI1586) of *H. influenzae* (20). These two ORFs were not analyzed further.

The *H. ducreyi* gmhA ORF was 585 nucleotides long (nucleotides 822 to 1406 in Fig. 2) and encoded a predicted protein with a calculated molecular mass of 21,302 Da (Fig. 2). BLAST searches of nonredundant DNA and protein databases revealed that the deduced amino acid sequence of the *H. ducreyi* GmhA protein was 87% identical and 93% similar to that of the GmhA protein of *H. influenzae* (8, 47) and 73% identical and 84% similar to that of the GmhA protein of *E. coli* (7).

Complementation of an *E. coli* gmhA mutant with the *H. ducreyi* gmhA gene homolog. The high degree of identity between the GmhA protein of *E. coli* and the predicted protein encoded by the *H. ducreyi* gmhA gene homolog led us to determine whether this similarity was reflected at the functional level. To accomplish this, the entire gmhA gene of *H. ducreyi* 35000 was amplified by PCR and inserted into cloning vector pCR2.1, yielding recombinant plasmid pCR253. This plasmid was used to electroporate *E. coli* x711, which has been shown to contain a chromosomal deletion of the gmhA gene (7). One of the resultant transformants [x711(pCR253)] was selected for further study.

The LPS molecules present in proteinase K-treated whole-cell lysates of the relevant *E. coli* strains were analyzed by Tricine-SDS-PAGE (Fig. 3). As expected from a recent study (7), the truncated LPS of strain x711 (Fig. 3, lane B) migrated much more rapidly in SDS-PAGE than did the LPS of strain x705 (Fig. 3, lane A), the immediate parent of strain x711 (14). The presence of vector pCR2.1 in x711 did not affect the rate of migration of the LPS (Fig. 3, lane C). In contrast, the LPS of x711(pCR253) (Fig. 3, lane D) migrated much more slowly, at a rate that was indistinguishable from that of the LPS of the x705 parent strain. This latter result indicated that the *H. ducreyi* gmhA gene was able to complement an *E. coli* gmhA mutant.

No recombinant *E. coli* strains containing pCR253 gave rise to the opaque and translucent colony variants originally observed with *E. coli* DH5α(pHD250). This result indicated that the genetic element(s) responsible for the opaque and trans-
lucent colony variation must be located in the remaining 16 kb of H. ducreyi DNA in pH250. Further analysis of this 16-kb region of H. ducreyi DNA was not performed in this study.

Construction of an isogenic H. ducreyi gmhA mutant. To confirm that the gmhA gene of H. ducreyi was involved in LOS biosynthesis, an isogenic gmhA mutant was constructed by allelic exchange. A cat cartridge was ligated into the PpuII restriction site of the gmhA gene in pH251 (Fig. 1). The resultant plasmid, pH252, was linearized by digestion with BsaI and used to electroporate H. ducreyi 35000. Ten chloramphenicol-resistant H. ducreyi transformants were tested initially by PCR (with oligonucleotide primers P1 and P2 [Fig. 2]) to detect the occurrence of allelic exchange involving the replacement of the wild-type gmhA gene with the mutated allele containing the cat cartridge. Nine of these 10 chloramphenicol-resistant transformants yielded two or more PCR products, consistent with a single crossover event. Only one of these transformants, strain 35000.252, yielded a single PCR product with the correct predicted size of 3 kb, representing the disrupted 1.6-kb gmhA gene containing the 1.4-kb cat cartridge.

Southern blot analysis was performed to confirm that strain 35000.252 was an isogenic gmhA mutant. When chromosomal DNA from wild-type parent strain 35000 was probed with a 330-bp fragment from the H. ducreyi gmhA gene (Fig. 1), a 2.7-kb AflIII fragment hybridized with this probe (Fig. 4, lane A). This same probe hybridized with a 4-kb AflIII fragment of chromosomal DNA from strain 35000.252 (Fig. 4, lane B). This result is consistent with the replacement of the wild-type gmhA gene with the mutated allele containing the 1.4-kb cat cartridge. When the cat cartridge was used as a probe, a 4-kb AflIII fragment from 35000.252 also hybridized with this cat cartridge (Fig. 4, lane D). Chromosomal DNA from wild-type parent strain 35000 failed to hybridize with the cat cartridge probe (Fig. 4, lane C).

Characterization of the isogenic gmhA mutant. Growth of wild-type strain 35000 and gmhA mutant 35000.252 in broth revealed that both strains grew at the same rates and to the same final densities (data not shown). The outer membrane of the gmhA mutant (Fig. 5, lane B) did not lack any proteins expressed by that of the wild-type parent strain (Fig. 5, lane A), although some major outer membrane proteins of these two strains differed in their relative abundances (Fig. 5, closed arrows). Two minor proteins present in the gmhA mutant (Fig. 5, open arrows) were absent in the wild-type parent strain; one of these may be the 26-kDa protein encoded by the cat gene.

SDS-PAGE followed by silver staining showed that the LOS molecules of strains 35000, 35000.7, and 35000.252 all migrated at different rates. The LOS of wild-type strain 35000 migrated at the lowest rate (Fig. 6, panel 1, lane A). The LOS of isogenic gmhA mutant 35000.252 (Fig. 6, panel 1, lane C) migrated the fastest, whereas the LOS of rfaK (lbgB) mutant 35000.7 (Fig. 6, panel 1, lane B) migrated at an intermediate rate. These results suggested that the oligosaccharide component of the LOS of the gmhA mutant was shorter than that of the rfaK (lbgB) mutant. In Western blot analysis with MAb 3E6, wild-type strain 35000 was used as the positive control and rfaK (lbgB) mutant 35000.7 was used as the negative control (60). MAb 3E6 readily bound the LOS of strain 35000 (Fig. 6, panel 2, lane A) but failed to bind the LOS of both rfaK (lbgB) mutant 35000.7 and gmhA mutant 35000.252 (Fig. 6, panel 2, lanes B and C, respectively).

Complementation of the H. ducreyi gmhA mutant. To eliminate the possibility that an undetected secondary mutation was responsible for the altered LOS phenotype of the H. ducreyi gmhA mutant, complementation analysis was performed. The wild-type H. ducreyi gmhA gene was cloned into pLS88,
yielding recombinant plasmid pLS253 (Fig. 1). Both the pLS88 vector and pLS253 were used to electroporate H. ducreyi gmhA mutant 35000.252. The presence of the vector alone in the H. ducreyi gmhA mutant [i.e., strain 35000.252(pLS88)] (Fig. 6, panels 1 and 2, lanes D) had no detectable effect on the migration rate or antigenic characteristics of the LOS relative to those of the LOS of strain 35000.252 (Fig. 6, panels 1 and 2, lanes C). In contrast, the presence of the wild-type H. ducreyi gmhA gene in trans in the gmhA mutant [i.e., strain 35000.252 (pLS253)] (Fig. 6, panels 1 and 2, lanes E) resulted in the expression of a LOS molecule which had a migration rate and an MAb 3E6 reactivity indistinguishable from those of the wild-type LOS (Fig. 6, panels 1 and 2, lanes A).

**Virulence expression by the gmhA mutant.** Isogenic gmhA mutant 35000.252 was tested for the ability to produce dermal lesions in the temperature-dependent rabbit model (49). In two independent experiments, mean lesion scores obtained with wild-type strain 35000 were consistently higher than those obtained with the gmhA mutant for all sampling times and inocula (Table 2, experiments A and B). These apparent differences in lesion scores were highly significant ($P < 0.0001$). On day 7 postinfection, viable H. ducreyi organisms were recovered only from lesions produced by wild-type strain 35000.

A third virulence test was performed to demonstrate that complementation with the wild-type H. ducreyi gmhA gene restored the virulence of the gmhA mutant. The gmhA mutant 35000.252(pLS88) containing the plasmid vector alone showed the same markedly decreased level of virulence as the original gmhA mutant, 35000.252 (Table 2, experiment C), whereas complemented gmhA mutant 35000.252(pLS253), with the wild-type H. ducreyi gmhA gene present in trans, had the same level of virulence as wild-type parent strain 35000 (Table 2, experiment C). No viable H. ducreyi organisms were recovered from the lesions produced by the gmhA mutant containing the vector alone. Viable H. ducreyi organisms were recovered at the same frequencies (i.e., five of eight rabbits) from lesions produced by both wild-type parent strain 35000 and complemented gmhA mutant 35000.252(pLS253). The identity of the H. ducreyi organisms recovered from lesions produced by strain 35000.252(pLS253) was confirmed by colony blot analysis with MAb 3E6, by antimicrobial resistance testing, and by Southern blotting (data not shown). This latter Southern blot analysis was performed to confirm that the wild-type gmhA gene in pLS253 had not been exchanged into the chromosome in place of the mutant allele.

**TABLE 2.** Lesion formation by wild-type, gmhA mutant, and complemented gmhA mutant strains of H. ducreyi in the temperature-dependent rabbit model$	extsuperscript{a}$

<table>
<thead>
<tr>
<th>Exp</th>
<th>Strain</th>
<th>Inoculum size (CFU)</th>
<th>Mean ± SD lesion score on day:</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>A</td>
<td>35000 (wild type)</td>
<td>$10^5$</td>
<td>3.38 ± 0.48</td>
<td>3.88 ± 0.33</td>
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<tr>
<td></td>
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<td>$10^5$</td>
<td>1.63 ± 0.48</td>
<td>1.25 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>35000 (wild type)</td>
<td>$10^4$</td>
<td>3.00 ± 0</td>
<td>3.00 ± 0</td>
</tr>
<tr>
<td></td>
<td>35000.252 (gmhA mutant)</td>
<td>$10^4$</td>
<td>1.25 ± 0.66</td>
<td>0.63 ± 0.48</td>
</tr>
<tr>
<td>B</td>
<td>35000</td>
<td>$10^6$</td>
<td>3.88 ± 0.33</td>
<td>3.88 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>35000.252</td>
<td>$10^5$</td>
<td>2.13 ± 0.60</td>
<td>1.63 ± 0.48</td>
</tr>
<tr>
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<td>3.13 ± 0.60</td>
<td>2.88 ± 0.78</td>
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<td>1.50 ± 0.71</td>
<td>0.88 ± 0.60</td>
</tr>
<tr>
<td>C</td>
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<td>4.00 ± 0</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>35000</td>
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<td>4.00 ± 0</td>
<td>3.88 ± 0.35</td>
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<td>3.75 ± 0.46</td>
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</tr>
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</table>

$^a$ Eight rabbits were used in each experiment.

$^b$ Calculated for the difference between wild-type and test strain lesion scores from both inoculum sizes and from all 3 days. In addition, complemented gmhA mutant 35000.252(pLS253) was significantly more virulent than the gmhA mutant containing the pLS88 vector alone ($P = 0.0001$).
Conservation of the gmhA gene among *H. ducreyi* strains. Southern blot analysis of 11 strains of *H. ducreyi* isolated in seven different countries was performed with a 330-bp fragment from the *gmhA* gene of *H. ducreyi* 35000 (Fig. 1) as a probe. Strains 35000, H09, H012, H013, 512, 1145, 1151, 1352, Cha-1, R018, and STD101 (60) all showed hybridization of a 2.7-kb AflII fragment of chromosomal DNA to the probe, indicating the presence of the *gmhA* gene in all 11 strains (data not shown).

**DISCUSSION**

It is well accepted that the LPS or LOS molecules present in the outer membranes of gram-negative pathogens play a critical role in disease caused by these organisms because of the inflammatory ability of these amphipathic molecules (48). Not unexpectedly, there is now ample evidence that the LOS of *H. ducreyi* is capable of inducing the formation of dermal lesions even in the absence of viable organisms (10, 65). Efforts to determine structure-function relationships of *H. ducreyi* LOS have been assisted by recent advances in knowledge about the physical structure of this molecule (1, 21, 37, 38, 56). However, still relatively little is known about what other role(s) LOS may play in the pathogenesis of chancroid beyond provoking a significant inflammatory response.

Mutant analysis of the LOSs of other pathogens, including *Neisseria gonorrhoeae* and *H. influenzae*, has been instrumental in establishing the relevance of the oligosaccharide component of LOS to the pathogenesis of the diseases caused by these organisms (13, 28, 39, 46, 47, 59). Considerable evidence derived from in vitro experiments has indicated that the oligosaccharide component of the LOS of *N. gonorrhoeae* contributes to the virulence of this mucosal surface pathogen (54, 55). Similarly, the use of relevant animal models has shown that the oligosaccharide component of *H. influenzae* type b LOS is essential for virulence expression by this encapsulated pathogen (13, 28, 31, 68, 69). More recently, mutant analysis was used to demonstrate the effect of changes in both the lipid A structure and the phosphorylation state of the LOS oligosaccharide on the virulence of nontypeable *H. influenzae* in an animal model (15).

The first indication that LOS oligosaccharide structure was important in the ability of *H. ducreyi* to produce disease was obtained over a decade ago (41, 42) in studies which compared different strains or variants of *H. ducreyi* for their virulence in a rabbit model. These experiments indicated that changes in the migration characteristics of the LOS in SDS-PAGE, which most likely reflected differences in LOS oligosaccharide composition, could be correlated with virulence differences in animals. Mutant analysis of the involvement of *H. ducreyi* LOS in virulence expression did not begin until relatively recently, when two different laboratories constructed *rfaK* (*lbgB*) mutants of *H. ducreyi* which expressed an LOS with a truncated oligosaccharide (9, 21, 60). Lack of expression of the *rfaK* (*lbgB*) gene product adversely affected the ability of *H. ducreyi* to both attach to and invade human keratinocytes in vitro (9, 21) but did not have a statistically significant effect on the ability of *H. ducreyi* to produce skin lesions in the temperature-dependent rabbit model (60).

It is particularly relevant to the present study that the first LOS mutant (originally designated I69) of *H. influenzae* type b which was shown to exhibit reduced virulence in the infant rat model (69) was subsequently found to express a LOS molecule comprised of only lipid A and a single phosphorylated 2-keto-3-deoxyoctulosonic acid residue (26). This LOS molecule, which lacked an oligosaccharide moiety, migrated much more rapidly in SDS-PAGE than did the LOS molecule of the wild-type parent strain (69). Subsequent studies revealed that the I69 mutant possessed a single-base-pair deletion in a previously unidentified gene, designated *ism* (47). Very shortly after this discovery, it was found that the *H. influenzae* *ism* gene was a homolog of the *E. coli* *gmhA* gene (previously designated *lpcA*), which encodes a phosphoheptose isomerase (7, 8). This enzyme catalyzes the conversion of sedoheptulose-7-phosphate into d-glycero-d-mannoheptose-7-phosphate (7), which is essential for the synthesis of the ADP-D-glycero-D-mannoheptose precursor involved directly in the synthesis of the oligosaccharide (17).

The *H. ducreyi* *gmhA* mutant characterized in the present study expressed a LOS that migrated more rapidly in SDS-PAGE than the LOS of the wild-type parent strain. Moreover, the LOS of this *H. ducreyi* *gmhA* mutant also migrated more rapidly than the truncated LOS expressed by an *rfaK* (*lbgB*) mutant (60). Consistent with this rapid migration phenotype in SDS-PAGE, the LOS of the *H. ducreyi* *gmhA* mutant lost reactivity with MAb 3E6, which is directed against a surface-exposed epitope of *H. ducreyi* LOS (25). Expression of the *H. ducreyi* *gmhA* gene product in trans in *E. coli* CH711, a strain with a *gmhA* deletion, resulted in the synthesis of an LOS which migrated at a rate indistinguishable from that of the LOS of the *E. coli* parent strain. This same *E. coli* *gmhA* mutant was also complemented by the wild-type *H. influenzae* *gmhA* gene (8). It can be inferred from these data that the *gmhA* mutation in *H. ducreyi* resulted in the expression of a LOS molecule with a severely truncated oligosaccharide moiety and that the product of the *H. ducreyi* *gmhA* gene is a homolog of both the *E. coli* and the *H. influenzae* GmahA proteins.

Interestingly, the *H. ducreyi* *gmhA* mutant exhibited some changes in the relative abundances of a few major outer membrane proteins (Fig. 5), similar to the changes described for deep rough LPS mutants of enteric bacteria (19, 34, 40). Whether these modest alterations in protein expression could have affected virulence expression by the *gmhA* mutant cannot be determined directly from the available data. However, an isogenic *mgm* mutant of *H. ducreyi* which lacks the ability to express one of the two OmpA-like major outer membrane proteins of this organism (33) was shown to be fully virulent in the temperature-dependent rabbit model (32a). This latter finding suggests that the observed changes in the relative abundances of the three outer membrane proteins in the *gmhA* mutant (Fig. 5) were not likely to have caused a reduction in virulence.

Southern blot analysis revealed that the *gmhA* gene is apparently well conserved among *H. ducreyi* strains. This finding is not surprising, especially in view of the fact that *gmhA* is known to be highly conserved among enteric bacteria (7). It is also interesting to note that the *H. ducreyi* *gmhA* gene, like the *rfaK* (*lbgB*) gene of this pathogen (21, 60), is not located within a cluster of other genes whose products are likely directly involved in LOS biosynthesis. The *H. ducreyi* *rfaK* (*lbgB*) gene is located adjacent to another gene (*lbgA*) whose expression is essential for the synthesis of wild-type LOS, but the *H. ducreyi* genes flanking the *lbgAB* genes do not encode proteins predicted to be involved in LOS expression (60). This situation is in contrast to the multiple, closely linked genes involved in enteric core LPS biosynthesis (i.e., the *E. coli* *rfa* genes) (32, 53). Similarly, the *H. influenzae* *gmhA* gene is not located among other LOS genes but instead is located between genes for two different ABC transport systems. Relative to *gmhA* in *H. influenzae* (47), several *dpp* genes essential for efficient dipeptide transport are located immediately upstream, whereas
several art gene homologs, which are likely involved in arginine uptake, are located immediately downstream.

Complementation analysis (Fig. 6) was used to confirm that the H. ducreyi gmhA mutant did not possess a secondary mutation(s) which could have affected LOS expression. Analysis of the growth rate of this mutant indicated that, in broth medium in vitro, the absence of gmhA gene expression did not affect detectably either the rate or the extent of growth. In contrast, the absence of the gmhA gene product had a pronounced effect on H. ducreyi in vivo. In the temperature-dependent rabbit model, the H. ducreyi gmhA mutant was markedly less able than the wild-type parent strain to produce dermal lesions (Table 2). In addition, this same mutant could not be recovered from lesions at 7 days postinfection, whereas viable cells of the wild-type parent strain were present in almost all of the lesions sampled. Restoration of GmhA expression, accomplished by provision of the wild-type H. ducreyi gmhA gene in trans, resulted in a level of virulence equivalent to that obtained with the wild-type parent strain.

It has been well established that H. influenzae type b LOS mutants with altered (i.e., shortened) oligosaccharide chains usually exhibit decreased virulence in the infant rat model (13, 28, 31, 68). In fact, the H. influenzae type b ism (i.e., I69) mutant is one of the least virulent of all of the LOS mutants tested to date (28). The H. ducreyi gmhA mutant in the present study was also less virulent than its wild-type parent strain. This significant diminution in the virulence of the H. ducreyi gmhA mutant is in marked contrast to the effect of the rfaK (lbgB) mutation on the virulence of H. ducreyi (60). The H. ducreyi rfaK (lbgB) mutant possessed a less truncated LOS (21) (relative to that of the gmhA mutant) and did not exhibit a statistically significant reduction in virulence, although there was an apparent trend in this direction (60). While it could be inferred from these data that the extent of truncation of the LOS oligosaccharide moiety, as evidenced by LOS migration rate in SDS-PAGE, might be inversely related to virulence potential in H. ducreyi, results derived from virulence testing of LOS mutants of H. influenzae type b indicated the inherent inaccuracy of such a generalization (13, 28). Determination of whether the lack of GmhA expression has an effect on the virulence of H. ducreyi for humans will necessarily have to await testing of the gmhA mutant in the human challenge model for experimental chancroid (57, 58).

In conclusion, we have identified the H. ducreyi gmhA gene homolog. Expression of the gmhA gene product was shown to be essential for the synthesis of wild-type LOS by H. ducreyi. Insertional inactivation of the gmhA gene had an adverse effect on the virulence of H. ducreyi 35000 in the temperature-dependent rabbit model for experimental chancroid. The availability of this cloned gene and the related isogenic H. ducreyi mutant, together with the rfaK (lbgB) mutants of H. ducreyi (21, 60), should facilitate future studies on structure-function relationships within the oligosaccharide portion of H. ducreyi LOS.

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REFERENCES


Hennessy, K. J., J. J. Landolo, and B. W. Fenwick. 1993. Serotype identifica-


Klena, J. D., E. Pradel, and C. A. Schnaitman. 1992. Comparison of lipo-


